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Improvement of a monopartite ecdysone receptor gene switch and demonstration of its utility in regulation of transgene expression in plants

Venkata S. Tavva[1,2], Subba R. Palli[1], Randy D. Dinkins[3] and Glenn B. Collins[2]

1 Department of Entomology, University of Kentucky, Lexington, KY, USA
2 Plant and Soil Sciences Department, University of Kentucky, Lexington, KY, USA
3 USDA-ARS Forage-Animal Production Research Unit, Lexington, KY, USA

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Correspondence
S. R. Palli, Department of Entomology, 1100 Nicholasville Road, University of Kentucky, Lexington, KY 40546-0091, USA
Fax: +1 859 323 1120
Tel: +1 859 257 4962
E-mail: rpalli@uky.edu

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In plants, regulation of transgene expression is typically accomplished through the use of inducible promoter systems. The ecdysone receptor (EcR) gene switch is one of the best inducible systems available to regulate transgene expression in plants. However, the monopartite EcR gene switches developed to date require micromolar concentrations of ligand for activation. We tested several EcR mutants that were generated by changing one or two amino acid residues in the highly flexible ligand-binding domain of *Choristoneura fumiferana* EcR (CfEcR). Based on the transient expression assays, we selected a double mutant, V395I + Y415E (VY), of CfEcR ($CfEcR_{VY}$) for further testing in stable transformation experiments. The $CfEcR_{VY}$ mutant only slightly improved the induction characteristics of the two-hybrid gene switch, whereas the $CfEcR_{VY}$ mutant significantly improved the induction characteristics of the monopartite gene switch ($VGCfE_{VY}$). The ligand sensitivity of the $VGCfE_{VY}$ switch was improved by 125–15 625-fold in different transgenic lines analyzed, compared to the $VGCfE_{Wt}$ switch. The utility of the $VGCfE_{VY}$ switch was tested by regulating the expression of an *Arabidopsis* zinc finger protein gene (*AtZFP11*) in both tobacco and *Arabidopsis* plants. These data showed that the $VGCfE_{VY}$ switch efficiently regulated the expression of *AtZFP11* and that the phenotype of AtZFP11 could be induced by the application of ligand. In addition, the affected plants recovered after withdrawal of the ligand, demonstrating the utility of this gene switch in regulating the expression of critical transgenes in plants.

Technology that provides control over transgene expression has several potential applications for both basic plant biology research and in production agriculture. In plants, control of transgene expression is commonly achieved through the use of an inducible promoter system that transactivates the transgene in response to an exogenous inducer. There are a number of circumstances in which it is advantageous to use an inducible gene regulation system [1,2], the most obvious being when introducing transgenes whose constitutive expression is detrimental or even lethal to the host plants [3]. Moreover, inducible gene expression systems provide more precise regulation and function of the target gene when compared to constitutive promoters.

Abbreviations
AD, activation domain; CfEcR, *Choristoneura fumiferana* ecdysone receptor; $CfEcR_{VY}$, double mutant, V395I + Y415E, of *Choristoneura fumiferana* ecdysone receptor; CH9, chimera 9; DBD, DNA-binding domain; EcR, ecdysone receptor; FMV, figwort mosaic virus; HsRXR, *Homo sapiens* retinoid X receptor; LBD, ligand-binding domain; LmRXR, *Locusta migratoria* retinoid X receptor; MMV, mirabilis mosaic virus; qRT-PCR, quantitative RT-PCR; RE, response element; RLU, relative light units; RXR, retinoid X receptor.

Among various inducible gene regulation systems available, chemical-inducible systems provide an essential tool for the control of *in vivo* transferred genes. During the past decade, several chemical-inducible gene expression systems have been developed for applications in plants [3–19]. The utility of such a system is determined mainly by there being undetectable expression of the transgene prior to application of the inducer chemical, and the induced gene expression levels being comparable to or higher than with a strong constitutive promoter such as the CaMV 35S promoter [14]. In addition, the optimal chemical-inducible system would employ an inexpensive, nontoxic inducer whose application can be fully controlled, that does not cause pleiotropic effects, that functions in a dose-dependent manner, and that ceases induction upon its removal [14]. Although several chemical-inducible gene expression systems have been described for plants, most inducers, including tetracycline, copper and steroid hormones, are not suitable for field applications, due to the nature of the chemicals and their possible effects on the environment [3,4,8,9,16,20–23]. The ethanol switch derived from the filamentous fungus *Aspergillus nidulans* has been shown to be useful in regulating transgene expression in several plant species, including tobacco, oilseed rape, tomato, and *Arabidopsis* [7,13,24–26]. Although ethanol can be used to regulate transgene expression under field conditions, the alcR/alcA system has some limitations under *in vitro* conditions [13,27].

Synthetic transcriptional activators have been developed for use in plant systems to induce gene expression in response to mammalian steroid hormones (dexamethasone and estradiol), and both steroidal and nonsteroidal agonists of the insect hormone 20-hydroxyecdysone [3,4,6,17,28–31]. The nuclear receptors used in monopartite gene switch format generally consist of a transcriptional activation domain fused to a DNA-binding domain (DBD) and a ligand-binding domain (LBD). The chimeric gene (transactivation domain–DBD–LBD) is expressed under the control of a constitutive promoter. In the presence of a specific ligand, the fusion protein translocates into the nucleus, binds the cognate response elements (REs), and transcriptionally activates the reporter gene (Fig. 1). LBDs from the ecdysone receptor (EcR) of *Drosophila melanogaster* [32,33], *Heliothis virescens* [30,31], *Ostrinia nubilalis* [2] and *Choristoneura fumiferana* [12] have been used to create EcR-based gene regulation systems for applications in plants. Among them, the *C. fumiferana* EcR-based system, which responds exclusively to nonsteroidal ecdysone agonists such as methoxyfenozide, was demonstrated to induce greater levels of transgene expression than the CaMV 35S promoter in transgenic tobacco and *Arabidopsis* plants [1,12]. All monopartite EcR-based gene switches developed to date require micromolar concentration of methoxyfenozide for activation of the transgene; 61.3–122 μM methoxyfenozide was required to activate a coat protein gene in transgenic *Arabidopsis* plants [1], 10–30 μM methoxyfenozide was required to activate reporter gene expression in transgenic tobacco and *Arabidopsis* plants [12], and 1200 mg of methoxyfenozide was required to induce *MS45* in maize [2]. This certainly limits the usefulness of these gene switches for large-scale applications.

Recently, we have developed a two-hybrid EcR gene switch with high ligand sensitivity and low background expression levels when compared to the earlier versions of EcR gene switches [14]. The chemical-inducible gene regulation system based on the two-hybrid gene switch requires three expression cassettes, two receptor expression cassettes, and one reporter or target gene expression cassette, as compared to the monopartite gene switch, which is composed of one receptor cassette and one reporter gene expression cassette (Fig. 1). In a two-hybrid switch format, the GAL4 DBD was fused to the LBD of the *C. fumiferana* ecdysone receptor (CfEcR), and the VP16 activation domain (AD) was fused to the LBD of *Locusta migratoria* retinoid X receptor (LmRXR) or *Homo sapiens* retinoid X receptor (HsRXR). The ligand sensitivity of the EcR gene switch was improved by using a CfEcR + LmRXR two-hybrid switch, and reduced background expression levels were achieved by using the CfEcR + HsRXR two-hybrid switch [14]. By using a chimera between the LmRXR and HsRXR LBDs as a partner of CfEcR, we were able to combine these two important aspects of the gene switch together and develop a tight EcR gene regulation system with improved ligand sensitivity and reduced background expression in the absence of chemical ligand [15]. Our previous studies [14,15] were focused on the optimization of the EcR partner, RXR, to improve the performance of the EcR gene switch. The present study was focused on manipulating EcR by testing different CfEcR mutants in both two-hybrid and monopartite switch formats.

We predicted that the sensitivity of the EcR gene switch could be improved by changing critical amino acid residues in the ligand-binding pocket of EcR, because the crystal structure of the *H. virescens* ecdysone receptor exhibited a highly flexible ligand-binding pocket [34]. Mutational analysis in the LBD of CfEcR showed that the ligand-binding pocket of this EcR is highly flexible and that a single amino acid substitu-

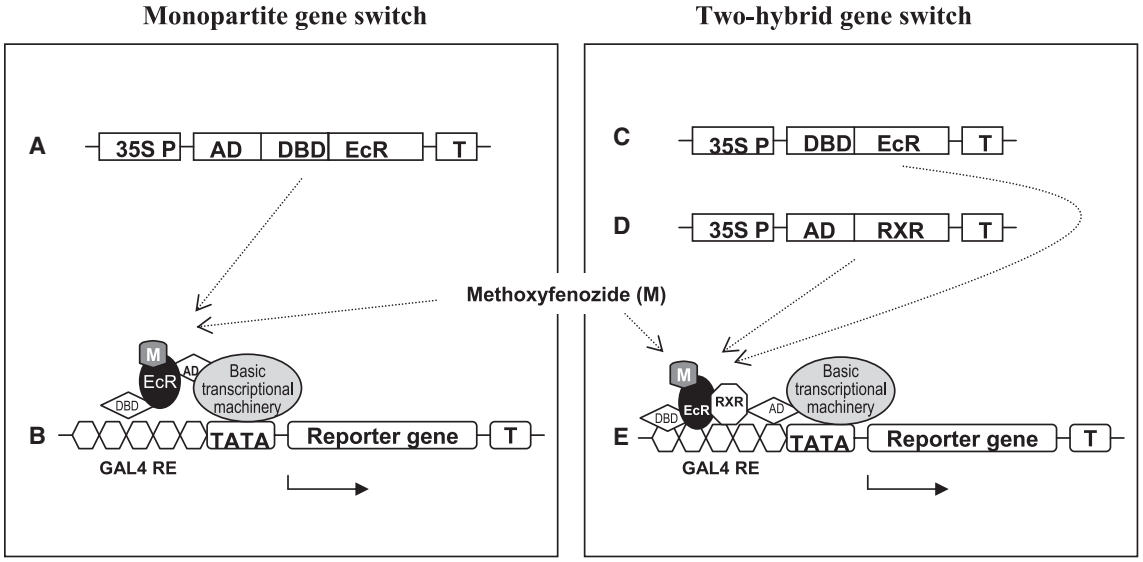


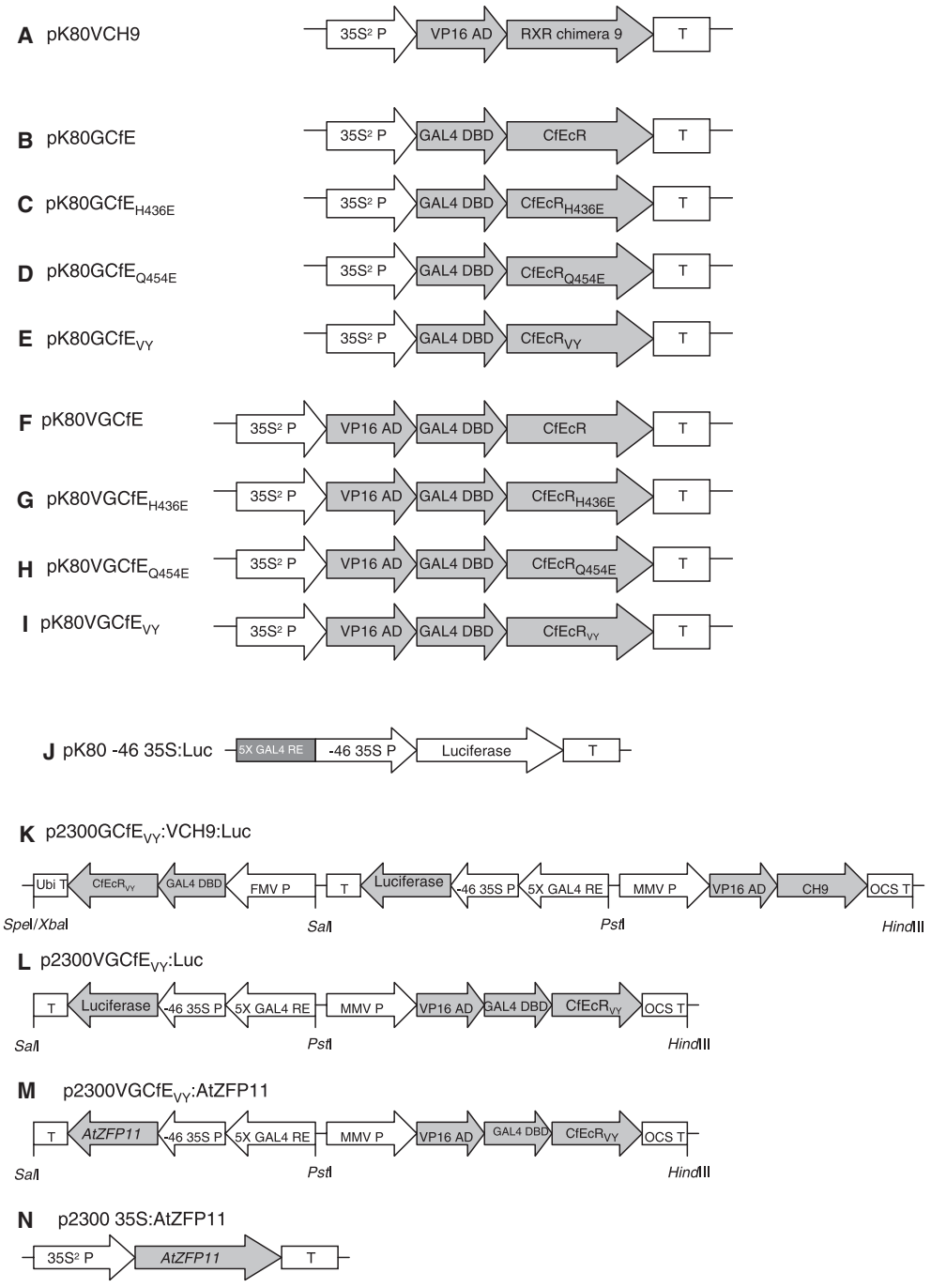
Fig. 1. Schematic representation of the chemical-inducible EcR gene regulation systems. Monopartite gene switch: the chimeric gene, AD:DBD:EcR LBD, is expressed under the control of a constitutive promoter (A). Upon addition of the ligand, methoxyfenozide (M), the fusion protein (AD:DBD:EcR) binds to five GAL4 REs located upstream of a minimal 35S promoter containing TATA box elements and transactivates the reporter gene expression (B). Two-hybrid gene switch: the chimeric genes, DBD:EcR LBD (C) and AD:RXR LBD (D) are under the control of constitutive promoters. The heterodimer of these fusion proteins transactivates the reporter gene placed under the control of five GAL4 REs and a minimal 35S promoter containing TATA box elements (E) in the presence of nanomolar concentrations of methoxyfenozide. The two-hybrid gene regulation system requires two receptor gene expression cassettes (DBD:EcR and AD:RXR), whereas the monopartite gene switch requires one receptor gene expression cassette (AD:DBD:EcR), to transactivate the reporter gene expression in the presence of methoxyfenozide. 35S P, a constitutive 35S promoter; AD, *Herpes simplex* transcription activation domain; DBD, yeast GAL4 DNA-binding domain; T, terminator sequence.

tion can result in significant changes in ligand binding, transactivation activity, and specificity [35,36]. Kumar *et al*. [35] demonstrated that substitution of alanine by proline at position 110 of the EcR from *C. fumiferana* resulted in loss of response to ecdysteroids, such as PonA and MurA, but not to synthetic nonsteroidal compounds, suggesting that the EcR-based gene expression system can be more tightly controlled by synthetic ecdysone agonists even in ecdysteroid-rich organisms. These studies, along with the other published reports [34,36], show the extreme flexibility and adaptability in the ligand-binding pocket of EcRs. Therefore, the present study was designed to screen several EcR mutants that were generated by changing one or two amino acids in the LBD of CfEcR. These EcR mutants were evaluated for their efficiency in transactivating transgene expression in both two-hybrid and monopartite gene switch formats by electroporating the plasmid DNA into tobacco protoplasts. On the basis of the transient expression studies, we selected a double mutant (V395I + Y415E) of CfEcR ($CfEcR_{VY}$) for additional stable transformation experiments to evaluate regulation of the expression of the luciferase reporter gene in both two-hybrid ($GCfE_{VY}$ + VCH9) and monopartite ($VGCfE_{VY}$) switch formats. In addition, we also tested the utility of the $VGCfE_{VY}$ switch in regulating the expression of a zinc finger protein transcription factor isolated from *Arabidopsis thaliana* (*AtZFP11*) in both *Arabidopsis* and tobacco plants.

Results

Selection of CfEcR mutants in transient expression studies

A screen of different EcR mutants generated by changing one or two amino acids in the LBD of CfEcR were carried out in a two-hybrid gene switch format to test their ability to induce luciferase reporter gene expression when placed under the control of GAL4 REs and a minimal 35S promoter. EcR mutants were coelectroporated with the constructs (Fig. 2) containing RXR chimera 9 (CH9) (pK80VCH9) and the luciferase reporter gene (pK80-46 35S:Luc) into tobacco protoplasts. The electroporated protoplasts were exposed to different concentrations of methoxyfenozide, and luciferase activity was measured 24 h after addition of



ligand (data not shown). Two single mutants, H436E (histidine at position 436 changed to glutamic acid) and Q454E (glutamine at position 454 changed to glutamic acid), and a double mutant, V395I + Y415E (VY; valine at position 395 and tyrosine at position 415 were changed to isoleucine and glutamic acid, respectively), of CfEcR that showed higher ligand sensitivity when compared to the wild-type EcR were selected for further analysis. These three mutants were used to carry out the methoxyfenozide dose–response study in both two-hybrid ($GCfE_{H436E}$ + VCH9, $GCfE_{Q454E}$ + VCH9, and $GCfE_{VY}$ + VCH9) and monopartite ($VGCfE_{H436E}$, $VGCfE_{Q454E}$, and $VGCfE_{VY}$) switch formats and compared to the data obtained from the gene switches containing wild-type CfEcR ($GCfE_{Wt}$ + VCH9 and $VGCfE_{Wt}$).

Fig. 2. Schematic representation of gene switch constructs. (A) The pK80VCH9 VP16 AD fusion of RXR CH9 was cloned into the pKYLX80 (pK80) vector. (B–E) GAL4 DBD fusions of the CfEcR LBD were cloned into the pK80 vector. pK80GCfE$_{Wt}$, pK80GCfE$_{H436E}$, pK80GCfE$_{Q454E}$ and pK80GCfE$_{VY}$, receptor constructs where the GAL4 DBD was fused to either wild-type (Wt) EcR or EcR containing either H436E or Q454E or VY mutations. (F–I) The pKYLX80 vector consists of a chimeric receptor gene where the CfEcR LBD was fused to the VP16 AD and GAL4 DBD. pK80VGCfE$_{Wt}$, pK80VGCfE$_{H436E}$, pK80VGCfE$_{Q454E}$, pK80VGCfE$_{VY}$: receptor constructs where the VP16 AD and GAL4 DBD was fused to either wild-type EcR LBD or EcR containing H436E or Q454E or VY mutations respectively. (J) pK80-46 35S:Luc: the reporter gene expression cassette was constructed by cloning the luciferase reporter gene under the control of a minimal promoter (–46 35S) and GAL4 REs. (K) p2300GCfE$_{VY}$:VCH9:Luc: T-DNA region of the pCAMBIA2300 binary vector showing the assembly of CfEcR$_{VY}$ (FMV:GCfE$_{VY}$: UbiT), CH9 (MMV P:VCH9:OCS T) and luciferase gene expression cassettes. (L) p2300VGCfE$_{VY}$:Luc: T-DNA region of the pCAMBIA2300 binary vector consists of an MMV promoter-driven CfEcR$_{VY}$ expression cassette (MMV P:VGCfE$_{VY}$:OCS T) and luciferase reporter gene expression cassette. (M) p2300VGCfE$_{VY}$:AtZFP11: T-DNA region of the pCAMBIA2300 binary vector showing the receptor (MMV P:VP16 AD:GAL4 DBD:CfEcR$_{VY}$:OCS T) and transgene (5× GAL4 RE:–46 35S:AtZFP11:rbcS T) expression cassettes. (N) p2300 35S:AtZFP11: T-DNA region of the binary vector showing the assembly of *AtZFP11* cloned under the control of the CaMV 35S promoter and rbcS terminator. 35S^2 P, a modified CaMV 35S promoter with duplicated enhancer region; rbcS T, Rubisco small subunit polyA sequence; FMV P, FMV promoter; Ubi T, ubiquitin 3 terminator; MMV P, mirabilis mosaic virus promoter; OCS T, *Agrobacterium tumefaciens* octopine synthase polyA.

Effect of CfEcR mutations on the performance of the two-hybrid gene switch

The CfEcR$_{H436E}$ and CfEcR$_{Q454E}$ mutants, when coelectroporated with RXR CH9 in a two-hybrid switch format, showed higher levels of background luciferase activity in the absence of ligand when compared to CfEcR$_{Wt}$. The background expression level of the luciferase reporter gene when coelectroporated with CH9 and the CfEcR$_{VY}$ double mutant was almost same as that of the background luciferase activity observed with CH9 and CfEcR$_{Wt}$ (Fig. 3A). The relative light units (RLU) per microgram of protein of luciferase reporter gene expression differed by several orders of magnitude between the three different EcR mutants tested in transient expression studies. The differences in luciferase activity observed with different EcR mutants in the absence of ligand are reflected in fold induction values (Fig. 3B). The background luciferase activity as well as the magnitude of induction was several times

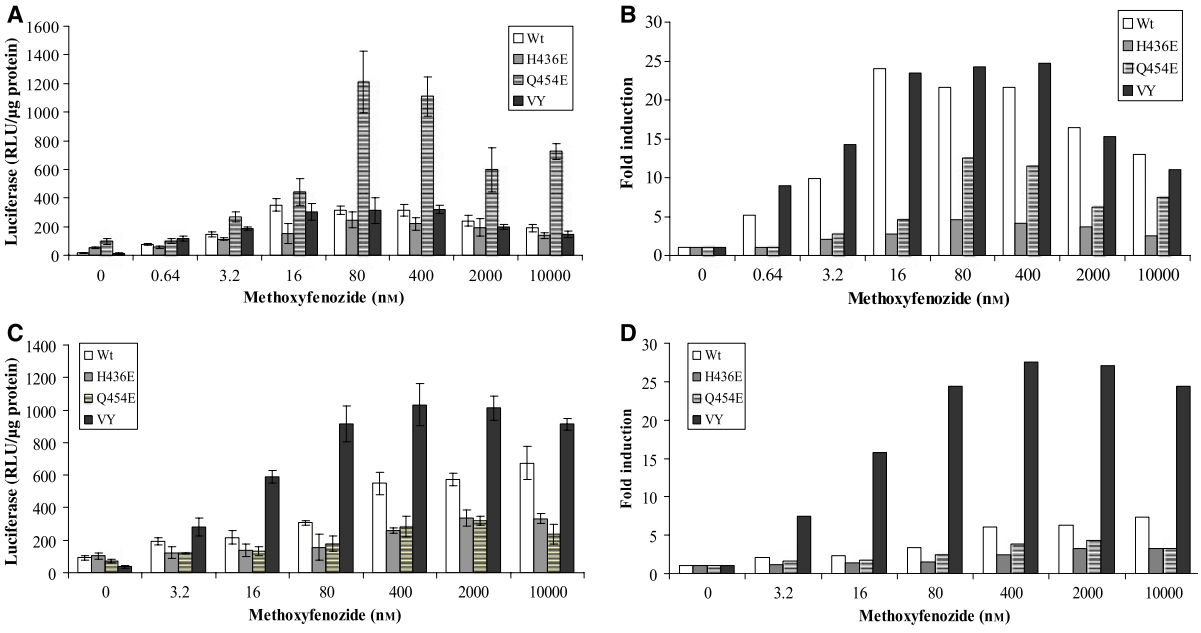


Fig. 3. Dose-dependent induction of the luciferase reporter gene by two-hybrid and monopartite gene switches. (A,B) Tobacco protoplasts were electroporated with pK80VCH9 plus pK80GCfE$_{Wt}$, pK80GCfE$_{H436E}$, pK80GCfE$_{Q454E}$ or pK80GCfE$_{VY}$ and reporter construct, and the electroporated protoplasts were incubated in growth media containing 0, 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM methoxyfenozide. (C,D) Tobacco protoplasts were electroporated with pK80VGCfE$_{Wt}$, pK80VGCfE$_{H436E}$, pK80VGCfE$_{Q454E}$ or pK80VGCfE$_{VY}$ and luciferase reporter construct, and then incubated in 0, 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM methoxyfenozide. The luciferase activity was measured after 24 h of incubation. RLU per microgram of protein shown are the mean of three replicates ± SD (A,C). Fold induction values (B,D) shown were calculated by dividing RLU·µg^{-1} protein in the presence of ligand with RLU·µg^{-1} protein in the absence of ligand.

higher with the $CfEcR_{Q454E}$ mutant than with either wild-type EcR or with any other EcR mutants tested. However, the luciferase reporter gene regulated by the two-hybrid switch containing the $CfEcR_{VY}$ mutant showed higher fold induction values than the the switches containing other EcR mutants. Of the three mutant EcRs tested in a two-hybrid gene switch format, the switch containing the $CfEcR_{VY}$ double mutant showed higher fold induction values. However, fold induction values obtained with the two-hybrid switch containing the $CfEcR_{VY}$ mutant were almost the same as the values obtained with $CfEcR_{Wt}$ when coelectroporated with CH9. Although the VY mutant of EcR was better than the other mutants tested, we did not find significant differences between the $CfEcR_{Wt}$ + CH9 and $CfEcR_{VY}$ + CH9 two-hybrid gene switches in terms of background expression and ligand sensitivity.

VY mutations improve the ligand sensitivity of the monopartite gene switch

Replacing $CfEcR_{Wt}$ with the $CfEcR_{H436E}$ and $CfEcR_{Q454E}$ single mutants did not improve the sensitivity and background expression levels of the monopartite gene switch (VGCfE). However, replacing $CfEcR_{Wt}$ with the $CfEcR_{VY}$ double mutant resulted in a significant improvement in the ligand sensitivity as well as background expression of the monopartite gene switch (Fig. 3C). The $CfEcR_{VY}$ mutant in a monopartite switch format ($VGCfE_{VY}$) resulted in low background levels of expression of the GAL4 RE-regulated luciferase reporter gene in the absence of ligand when compared to the monopartite switches containing either $CfEcR_{Wt}$ or the $CfEcR_{H436E}$ or $CfEcR_{Q454E}$ mutants (Fig. 3C).

The ligand sensitivity of the monopartite switch was improved 25-fold by using the $CfEcR_{VY}$ mutant as compared to $CfEcR_{Wt}$. The $VGCfE_{VY}$ gene switch induced luciferase activity that reached peak levels at 80 nM methoxyfenozide as compared to the $VGCfE_{Wt}$ switch, where the maximum luciferase activity (seven-fold) was observed at 10 000 nM methoxyfenozide. Moreover, at all methoxyfenozide concentrations tested, the fold induction values observed were higher with the $VGCfE_{VY}$ switch than with the $VGCfE_{Wt}$, $VGCfE_{H436E}$ or $VGCfE_{Q454E}$ monopartite gene switches (Fig. 3D).

VY mutations improve the performance of the two-hybrid and monopartite switches in transgenic *Arabidopsis* plants

The LBD of CfEcR containing the VY mutations ($GCfE_{VY}$) was cloned into a binary vector along with VP16:CH9 (VCH9) and luciferase expression cassettes to generate a two-hybrid gene switch (p2300$GCfE_{VY}$:VCH9:Luc) and $VGCfE_{VY}$ and luciferase expression cassettes to provide a monopartite gene switch (p2300$VGCfE_{VY}$:Luc) for transformation into *Arabidopsis*. T_2 seeds collected from five independent lines for two-hybrid and monopartite switches were plated on agar media supplemented with 50 $mg \cdot L^{-1}$ kanamycin and 0 (dimethylsulfoxime), 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM methoxyfenozide. After 20 days, three seedlings from each plate were collected and assayed separately for luciferase activity.

In the five T_2 *Arabidopsis* lines containing a two-hybrid ($GCfE_{VY}$:VCH9) gene switch, the level of luciferase reporter gene expression in the absence of methoxyfenozide was indistinguishable from the background readings detected in the transgenic plants that were transformed with a two-hybrid gene switch containing wild-type EcR ($GCfE_{Wt}$:VCH9) [15]. In all five lines tested, luciferase activity began to increase at the lowest concentration (0.64 nM) of methoxyfenozide and reached maximum levels at 3.2 or 16 nM, except in line 1, where luciferase induction reached peak levels with the application of 80 nM methoxyfenozide (Fig. 4A). Although there was no significant difference between the ligand sensitivities of the $GCfE_{Wt}$ + VCH9 and $GCfE_{VY}$ + VCH9 gene switches in the transient expression studies (Fig. 3A,B), we did observe significant differences in ligand sensitivity between these two gene switches in transgenic *Arabidopsis* plants. With employment of the $GCfE_{VY}$ + VCH9 two-hybrid gene switch, the luciferase reporter gene reached peak levels at 3.2–16 nM methoxyfenozide, as compared to the $GCfE_{Wt}$ + VCH9 switch, which required 16–80 nM methoxyfenozide to reach maximum levels [15].

As compared to the $VGCfE_{Wt}$ transgenic plants, the plants that were transformed with the $VGCfE_{VY}$ monopartite switch showed a significant increase in ligand sensitivity and a conspicuous reduction in the background reporter gene expression levels in the absence of ligand. As shown in Fig. 4B, the $VGCfE_{Wt}$ gene switch plants showed maximum luciferase activity at 10 000 nM methoxyfenozide. In all five $VGCfE_{VY}$ lines tested, the maximum luciferase activity was observed at 0.64–80 nM methoxyfenozide. The maximum induction of luciferase gene activity observed in different *Arabidopsis* lines transformed with the $VGCfE_{vy}$ switch construct was 3.7–6.8 times higher than the luciferase activity observed in the constitutively expressing 35S:Luc plants (Fig. 4B).

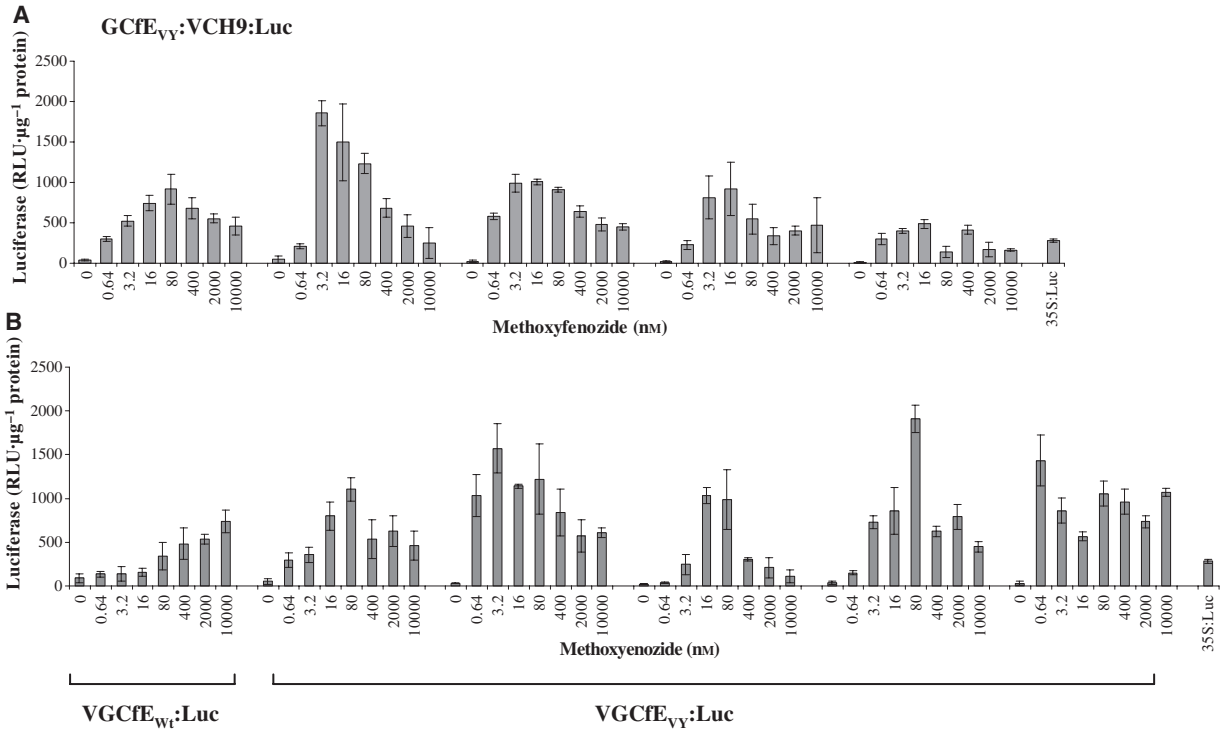


Fig. 4. Methoxyfenozide dose–response study with T2 *Arabidopsis* plants. Seeds collected from five transgenic lines for each construct, p2300GCfE$_{VY}$:VCH9:Luc (A) and p2300VGCfE$_{VY}$:Luc (B), were plated on agar media containing different concentrations of methoxyfenozide. Luciferase activity was measured in the seedlings collected at 20 days after plating the seeds on the induction medium. Luciferase activity in terms of RLU·µg^{-1} protein shown is the average of three replicates ± SD. The luciferase induction data collected from transgenic *Arabidopsis* plants developed for the p2300VGCfE$_{Wt}$:Luc construct are also shown in (B). 35S:Luc represents the average luciferase activity collected from five independent *Arabidopsis* plants developed for the p230035S:Luc construct. GCfE$_{VY}$:VCH9:Luc, VGCfE$_{VY}$:Luc and VGCfE$_{Wt}$:Luc: data collected from the plants that were transformed with p2300GCfE$_{VY}$:VCH9:Luc, p2300VGCfE$_{VY}$:Luc and p2300VGCfE$_{Wt}$:Luc constructs respectively.

Stable transformation of *Arabidopsis* and tobacco plants using the p2300VGCfE$_{VY}$:AtZFP11 construct

The expression levels of the *A. thaliana* zinc finger protein gene (*AtZFP11*) in wild-type control *Arabidopsis* plants are extremely low, and no mutant phenotype is presently associated with this gene. This AtZFP11 protein caused mortality and a deformed phenotype when overexpressed under the control of a CaMV 35S promoter in both *Arabidopsis* and tobacco [37]. There was difficulty in recovering healthy transgenic plants, and the seeds collected from the transgenic tobacco expressing *AtZFP11* under the CaMV 35S promoter failed to germinate on agar plates supplemented with kanamycin [37] (V. S. Tavva, unpublished results). Therefore, *AtZFP11* is an ideal candidate for testing the efficiency of the new monopartite EcR gene switch (VGCfE$_{VY}$) in plants.

We generated approximately 30 transgenic lines of each tobacco and *Arabidopsis* plant using the p2300VGCfE$_{VY}$:AtZFP11 construct (Fig. 2M). Fewer than 10% of the transgenic lines displayed an abnormal phenotype in the absence of methoxyfenozide, and the majority of the transformants grew well in the greenhouse. Seeds were obtained from the majority of the transgenic lines; the T_2 seedlings were tested for inheritance of the transgene by Southern blot analysis, and the levels of receptor gene expression were tested at the RNA level by northern blot analysis (data not shown). To test the methoxyfenozide-mediated induction of the *AtZFP11* transgene and associated phenotype, at least three independent transgenic lines each in *Arabidopsis* and tobacco were subjected to methoxyfenozide in a dose–response study. T_2 *Arabidopsis* and tobacco seeds were plated on agar media supplemented with kanamycin and different doses of methoxyfenozide.

Both *Arabidopsis* and tobacco transgenic plants expressing the *AtZFP11* gene under the control of the VGCfE$_{VY}$ monopartite switch showed no phenotypic differences from wild-type control plants when grown on media containing dimethylsulfoxime only (Figs 5A

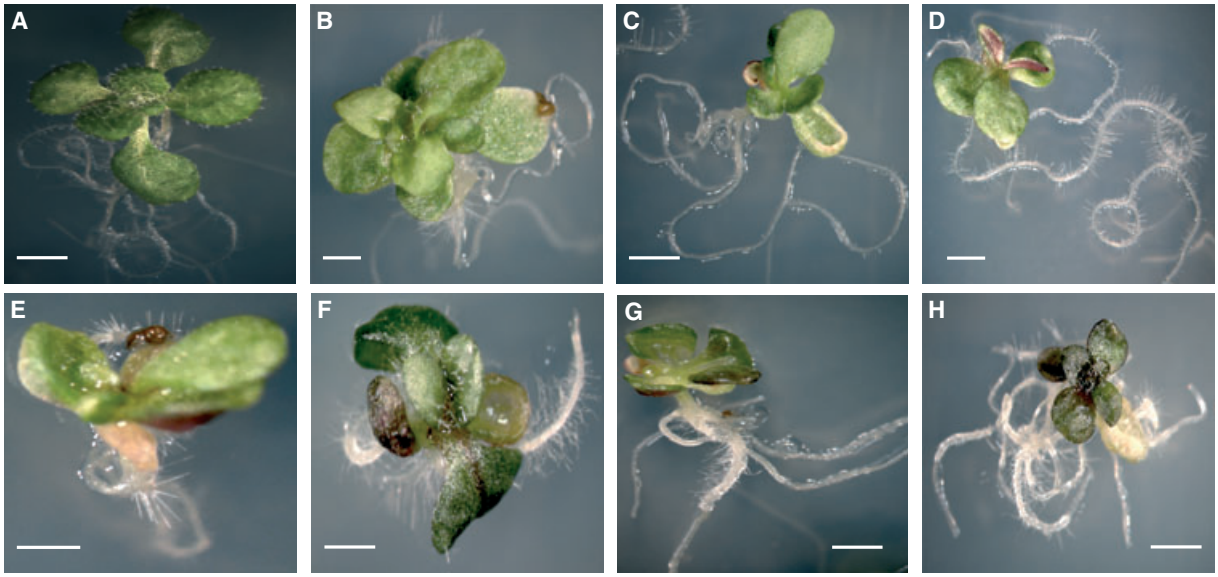


Fig. 5. Methoxyfenozide-inducible AtZFP11 phenotype in *Arabidopsis* seedlings. Transgenic *Arabidopsis* seedlings expressing *AtZFP11* under the control of the $VGCfE_{VY}$ monopartite gene switch. Pictures were taken 20 days after plating the seeds on agar media containing different methoxyfenozide concentrations. (A–H) Micrographs of the T2 transgenic *Arabidopsis* seedlings subjected to different methoxyfenozide treatments: (A) 0 nM (dimethylsulfoxime); (B) 16 nM; (C) 80 nM; (D) 400 nM; (E,F) 2000 nM; (G,H) 10 000 nM. Bars = 1 mm.

and 6A). The transgenic plants displayed an altered phenotype within 10 days of seed germination on the media containing as little as 16 nM methoxyfenozide (Figs 5 and 6). The AtZFP11-induced phenotype was more conspicuous at higher doses of methoxyfenozide, and no such phenotypes were observed in either *Arabidopsis* or tobacco seedlings grown on agar media without methoxyfenozide (Figs 5 and 6). Roots were thicker, rigid

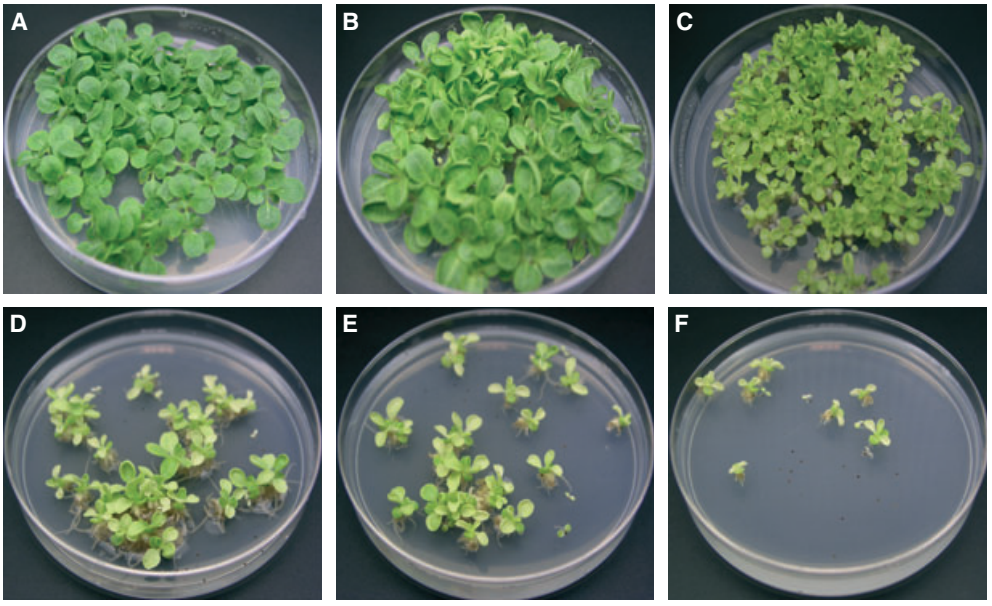


Fig. 6. Methoxyfenozide-inducible AtZFP11 phenotype in tobacco seedlings. Transgenic tobacco seedlings expressing *AtZFP11* under the control of the $VGCfE_{VY}$ monopartite gene switch and methoxyfenozide. Seeds collected from the T2 transgenic tobacco plant developed for the p2300$VGCfE_{VY}$:AtZFP11 construct were plated on agar media containing 300 $mg \cdot L^{-1}$ kanamycin and different concentrations of methoxyfenozide. Pictures were taken 1 month after plating the seeds on different methoxyfenozide concentrations: (A) 0 nM (dimethylsulfoxime); (B) 16 nM; (C) 80 nM; (D) 400 nM; (E) 2000 nM; (F) 10 000 nM.

and branched, and the plants had green and shrunken leaves, when compared to wild-type tobacco plants. We have observed similar growth defects with transgenic lines expressing *AtZFP11* under the 35S promoter [37]. To determine whether or not the transgenic plants could recover from the induced phenotype, tobacco seedlings that were grown on inducing medium for 1 month were transferred to fresh agar medium without methoxyfenozide. When maintained on agar plates without methoxyfenozide, tobacco seedlings that were transferred from the plates containing 16, 80, 400 or 2000 nM methoxyfenozide started recovering from the induced phenotype (Fig. 7). Plants subjected to 10 000 nM methoxyfenozide treatment recovered slowly from the induced phenotype after 1 month following removal of the ligand (Fig. 7).

Quantitative RT-PCR (qRT-PCR) analysis of methoxyfenozide-inducible *AtZFP11* expression level

To further analyze methoxyfenozide-inducible *AtZFP11* expression, *AtZFP11* mRNA levels were quantified using qRT-PCR in both *Arabidopsis* and tobacco seedlings that were subjected to different methoxyfenozide treatments and compared with CaMV 35S:AtZFP11-overexpressing plants and wild-type control plants. Low *AtZFP11* mRNA levels were observed in both *Arabidopsis* and tobacco transgenic plants constitutively expressing *AtZFP11* under the 35S promoter (Fig. 8A,B). This is presumably due to *AtZFP11* causing mortality and a deformed phenotype. We had difficulty in recovering both *Arabidopsis* and tobacco 35S:AtZFP11-expressing lines. Both *Arabidopsis* and tobacco transgenic plants showed low *AtZFP11* expression in the absence of ligand, and induced expression levels were higher than the levels detected in transgenic plants where *AtZFP11* was placed under the control of the 35S promoter (Fig. 8). The maximum induction of *AtZFP11* expression was observed at 80 nM methoxyfenozide in *Arabidopsis* and at 16 nM methoxyfenozide in tobacco. A correlation between the severity of the phenotype and expression levels of the *AtZFP11* transgene was noted. The *AtZFP11* level began to decrease in plants treated with more than 80 nM methoxyfenozide.

The endogenous *AtZFP11* expression in wild-type control *Arabidopsis* seedlings was extremely low (4.24×10^3 copies of AtZFP11·μg^{-1} of total RNA). In 35S:AtZFP11 *Arabidopsis* plants, the average *AtZFP11* mRNA level observed was 2.98×10^5 copies·μg^{-1} of total RNA, which is 70.3-fold higher than the *AtZFP11* mRNA level observed in the wild-type control plants (Fig. 8A). In transgenic *Arabidopsis* plants where *AtZFP11* was under the control of the $VGCfE_{VY}$ switch, the *AtZFP11* mRNA levels recorded in the plants treated with 80 nM methoxyfenozide were 6.1-fold and 429.2-fold higher than in the 35S:AtZFP11-overexpressing plants and wild-type *Arabidopsis* plants, respectively (Fig. 8A).

qRT-PCR analysis of RNA isolated from the tobacco plants expressing *AtZFP11* under the control of the $VGCfE_{VY}$ gene switch revealed that *AtZFP11* expression reached a peak level at 16 nM methoxyfenozide, and this accounts for a 30.55-fold increase over the *AtZFP11* mRNA levels observed in dimethylsulfoxime-treated plants. The *AtZFP11* mRNA levels observed in tobacco plants treated with 16 nM methoxyfenozide were 42.35-fold higher than the *AtZFP11* levels observed in the tobacco plants expressing *AtZFP11* under the control of the 35S promoter (Fig. 8B). Furthermore, *AtZFP11* expression levels went down after the $VGCfE_{VY}$ switch reverted to the uninduced state (Fig. 8B). The qRT-PCR data confirmed the reduction in AtZFP11 expression levels upon withdrawal of the ligand, and within 15 days the mRNA levels went down in the seedlings that were transferred from different methoxyfenozide treatments to medium containing no methoxyfenozide (Fig. 8).

Discussion

The two major findings presented in this article are the improved EcR monopartite switch and the demonstration of its utility in regulating the expression of transcription factor in plants. The ability to tightly regulate gene expression in plants is an essential tool for the elucidation of gene function. In order to regulate the expression of transgenes in plants, a number of inducible systems have been developed [3–19]. However, most of the systems are induced by compounds that are not suitable for agricultural use [3,4,8,9,16, 20–23]. The EcR-based gene switch is one of the best gene regulation systems available, because the chemical ligand, methoxyfenozide, required for its regulation is already registered for field use [38]. EcR has been used in several inducible gene regulation systems to control transgene expression in mammalian cells, transgenic animals, and plants [39]. The EcR gene switches described to date are mostly in monopartite format, require high concentrations of chemical ligand for induction, and show high background activity of the reporter or transgene in the absence of ligand [1,2,12,30,31].

We have previously demonstrated the utility of a two-hybrid EcR gene regulation system that has a lower background activity in the absence of ligand

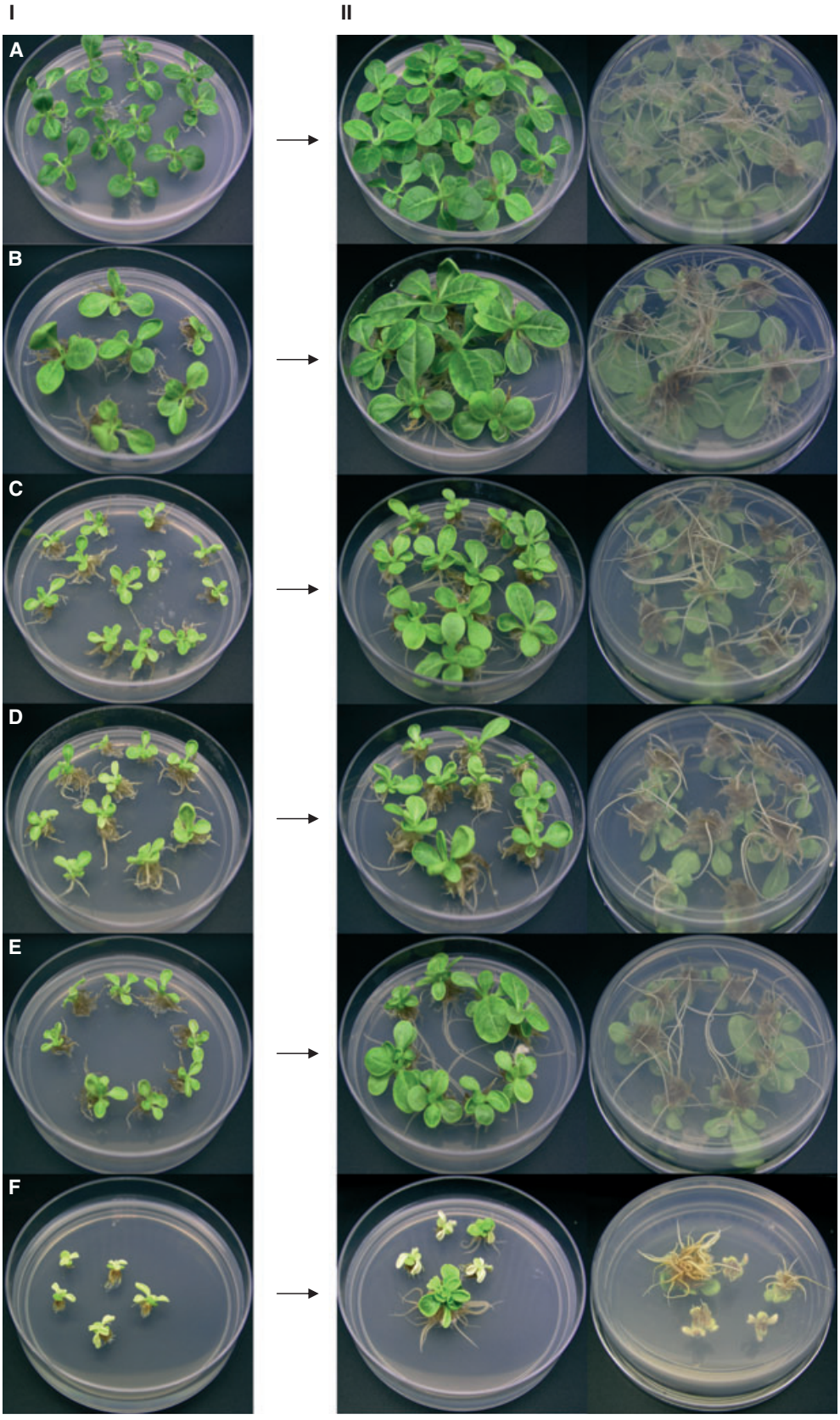

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Fig. 7. Transgenic tobacco seedlings showing the recovery of induced phenotype. (I) Tobacco seedlings that were growing on different concentrations of methoxyfenozide were transferred to fresh agar medium containing 300 mg·L^{-1} kanamycin, without any added inducer. Pictures were taken immediately after transfer onto the fresh medium. (II) Tobacco seedlings started showing the wild-type phenotype at 15 days after withdrawal of ligand. (A) 0 nM (dimethylsulfoxime); (B) 16 nM; (C) 80 nM; (D) 400 nM; (E) 2000 nM; (F) 10 000 nM.

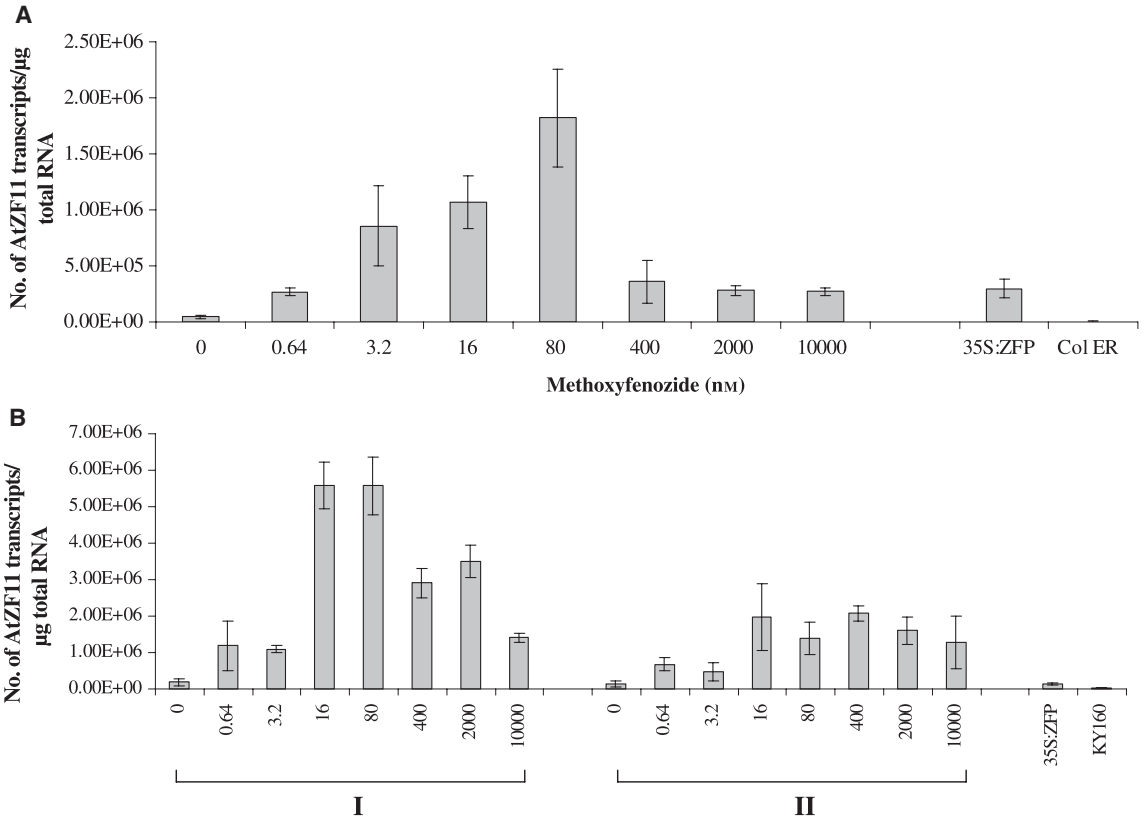

Fig. 8. Expression of *AtZFP11* in transgenic *Arabidopsis* and tobacco plants. The values in the histogram represent the *AtZFP11* expression levels adjusted to α-tubulin across all samples. Units are given as number of *AtZFP11* molecules·µg^{-1} of total RNA. Data represent an average of three replicates ± SD. (A) Graph showing *AtZFP11* expression levels in *Arabidopsis* seedlings grown for 20 days on dimethylsulfoxime, and 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM methoxyfenozide. This graph also shows *AtZFP11* expression levels in 35S:AtZFP11 *Arabidopsis* plants and wild-type control plants (Col ER). (B) Graph showing *AtZFP11* expression levels in tobacco seedlings grown for 1 month on dimethylsulfoxime, and 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM methoxyfenozide (I) and 15 days after removal of the ligand (II). This graph also shows the *AtZFP11* expression levels in transgenic tobacco developed for the construct where the *AtZFP11* gene was cloned under the control of a 35S promoter (35S:AtZFP11) and wild-type control plants (KY160).

and increased sensitivity and higher magnitude of induction as compared to the monopartite EcR gene switch [14,40]. In our earlier studies [14,15], we focused on the EcR partner, RXR, to optimize the CfEcR-based gene regulation systems for applications in plants. In the present study, we attempted to optimize CfEcR by screening different EcR mutants. To this end, we utilized the CfEcR homology model developed by Kumar *et al.* [35], where they identified 17 amino acids that were critical for 20-hydroxyecdysone binding. Mutational analysis at these 17 amino acids in transactivation assays resulted in the identification of EcR mutants that were better than wild-type EcR in terms of ligand sensitivity and transactivation ability [35].

We screened several EcR mutants, selected three mutants [H436E; Q454E; V395I + Y415E (VY)], and performed dose–response studies both in two-hybrid and in monopartite gene switch formats (Fig. 3). These studies showed that the $CfEcR_{VY}$ mutant

containing a monopartite switch showed a significant improvement in induction characteristics when compared to the switch containing wild-type EcR. Low background expression levels in the absence of ligand and high induced expression in the presence of nanomolar concentrations of methoxyfenozide were supported by the $VGCfE_{VY}$ monopartite switch (Fig. 3C). The monopartite $VGCfE_{Wt}$ switch requires micromolar concentrations of ligand for the activation of genes, and it does not support higher induction values as compared to the two-hybrid gene switch [14]. All previous studies utilizing the monopartite gene switch composed of EcR from *H. virescens* [30,31], *O. nubilalis* [2] or *C. fumiferana* [1,12] have required micromolar concentrations of the chemical ligand to transactivate target gene expression. However, the monopartite switch with the $CfEcR_{VY}$ mutant requires only nanomolar concentrations of ligand for transactivation of the luciferase reporter gene, both in transient expression studies using tobacco protoplasts, and in transgenic *Arabidopsis* plants (Figs 3 and 4). Transient assays with the monopartite gene switch constructs containing the $CfEcR_{VY}$ mutant showed maximum luciferase reporter gene activity in the presence of 80 nM methoxyfenozide, as compared to the monopartite gene switch containing $CfEcR_{Wt}$, where maximum luciferase levels were observed with the application of 10 μM methoxyfenozide (Fig. 3C). On the other hand, dose-dependent induction of luciferase activity in transgenic *Arabidopsis* plants developed for the p2300$VGCfE_{VY}$:Luc construct revealed that the maximum luciferase expression levels could be observed with the application of as little as 0.64–80 nM methoxyfenozide, depending on the transgenic line analyzed (Fig. 4B). On the basis of the transient expression studies, the sensitivity of the $VGCfE_{VY}$ switch is 25 times higher than that of the $VGCfE_{Wt}$ switch (Fig. 3D). The sensitivity of the $VGCfE_{VY}$ switch has been improved by 125–15 625-fold in the transgenic *Arabidopsis* plants analyzed as compared to the transgenic *Arabidopsis* plants containing the $VGCfE_{Wt}$ switch (Fig. 4B). These results suggest that mutations at amino acid positions 395 and 415 in the LBD of CfEcR can be used to improve the sensitivity and lower the background reporter gene expression of the monopartite gene switch as compared to the wild-type EcR.

To assess the usefulness of the $VGCfE_{VY}$ switch for applications in plants, we cloned *AtZFP11* under control of the EcR gene switch and introduced it into both *Arabidopsis* and tobacco plants. Overexpression of *AtZFP11* under the CaMV 35S promoter in tobacco resulted in severely reduced stem elongation, abnormal leaf shape and sterility, as described previously [37]. We also had difficulty in recovering *Arabidopsis* transgenic plants expressing *AtZFP11* under the 35S promoter, as these plants were severely deformed and dwarfed and did not set seed (data not shown). Molecular genetic approaches such as antisense RNA, loss of function, gain of function, ectopic expression and overexpression cannot be easily applied to genes that control fundamental processes of plant growth, differentiation, and reproduction [41].

Both *Arabidopsis* and tobacco transgenic plants developed for the p2300$VGCfE_{VY}$:AtZFP11 construct exhibited the methoxyfenozide-inducible AtZFP11 phenotype. The induced phenotype observed in these plants is similar to the phenotype observed with 35S:AtZFP11-expressing plants, confirming that the controlled expression of *AtZFP11* is necessary to recover healthy transgenic plants. Despite the severity of the induced AtZFP11 phenotype, we did not observe any differences in development and appearance between noninduced gene switch plants regulating the *AtZFP11* transgene and wild-type control plants. The induced expression of *AtZFP11* achieved was several times higher than the constitutive expression mediated by the CaMV 35S promoter (Fig. 8). The system is very sensitive to methoxyfenozide, with induction being observed even with the application of 0.64 nM methoxyfenozide. In addition, the induction of AtZFP11 was shown to be reversible in transgenic tobacco plants (Figs 7 and 8). Moreover, in tobacco seedlings, *AtZFP11* transcript levels declined upon withdrawal of ligand, and plants began to revert to the normal phenotype (Figs 7 and 8).

In summary, we demonstrated that the change in two amino acids in the LBD of CfEcR resulted in a complete change in ligand sensitivity and background activity of the monopartite gene switch. The system is very sensitive, and reporter gene induction was observed with nanomolar concentrations of methoxyfenozide, with reduced background expression levels similar to that of the two-hybrid gene switch, where the LmRXR or Hs–LmRXR chimera (CH9) was used as a partner for CfEcR in inducing the transgene expression [14,15]. The $VGCfE_{VY}$ switch is also very effective in both *Arabidopsis* and tobacco transgenic plants in regulating expression of *AtZFP11* (Figs 5–8). With this improvement in sensitivity and inducibility, the new monopartite gene switch containing the $CfEcR_{VY}$ mutant provides a new tool for regulating a variety of genes in plants.

Experimental procedures

DNA manipulations

For transient studies, the EcR (GAL4 DBD:CfEcR), RXR (VP16 AD:CH9) and reporter (−46 35S:Luc) gene expression cassettes were cloned in the pKYLX80 vector as described earlier [14]. The RXR CH9 containing helices 1–8 from HsRXR and helices 9–12 from LmRXR was used as a partner for CfEcR in a two-hybrid gene switch. The DNA sequence coding for the fusion protein of VP16 AD and RXR CH9 was transferred from the pVP16RXR chimera construct as described in Tavva *et al.* [15]. The EcR mutants were prepared as described in Kumar *et al.* (2002). The D, E and F domains of CfEcR, both wild-type and mutants [H436E; Q454E; and V395I + Y415E (VY)] were cloned downstream of the GAL4 DBD sequence in the pM vector (BD Biosciences Clontech, San Jose, CA, USA). The fusion gene, GAL4 DBD:CfEcR, was excised from the pM vector as an *Nhe*I–*Xba*I fragment and cloned into the pKYLX80 vector. The monopartite receptor expression cassette, VGCfE (VP16 AD:GAL4 DBD:CfEcR), was constructed by cloning the GAL4 DBD:CfEcR from pM vector into pVP16 vector. The resultant vector was restricted with *Nhe*I and *Xba*I and cloned into the VP16 AD:GAL4 DBD:CfEcR fusion gene in the pKYLX80 vector. The resulting constructs for the two-hybrid gene switch were designated as pK80VCH9, pK80GCfE$_{Wt}$, pK80GCfE$_{H436E}$, pK80GCfE$_{Q454E}$, and pK80GCfE$_{VY}$, and for the monopartite gene switch were designated as pK80VGCfE$_{Wt}$, pK80VGCfE$_{H436E}$, pK80VGCfE$_{Q454E}$ and pK80VGCfE$_{VY}$ (Fig. 2A–I). The reporter construct (pK80-46 35S:Luc) was generated by cloning the luciferase gene under the control of a CaMV 35S minimal promoter (−46 to +5 bp) and five copies of the GAL4 REs (Fig. 2J).

For the construction of a binary vector for plant transformation, the GAL4 DBD:CfEcR fusion gene was cloned under the FMV (figwort mosaic virus) promoter and Ubi (ubiquitin 3) terminator sequence, and the VP16 AD:CH9 fusion gene was cloned under the MMV (mirabilis mosaic virus) promoter and OCS (*Agrobacterium tumefaciens* octopine synthase) polyA sequences. The FMV and MMV promoter-driven expression cassettes were assembled into pSL301 vectors. The reporter and receptor expression cassettes were excised with appropriate restriction enzymes and assembled into the pCAMBIA2300 vector (CAMBIA, Canberra, Australia) for plant transformation. The binary vectors constructed for two-hybrid and monopartite gene switches were designated as p2300CfE$_{VY}$:CH9:Luc and p2300VGCfE$_{VY}$:Luc respectively (Fig. 2K,L).

Construction of p2300VGCfE$_{VY}$:AtZFP11

The *AtZFP11* sequence was amplified from cDNA prepared from the total RNA isolated from *Arabidopsis* seedlings. Oligonucleotide primers were synthesized to include the restriction enzyme *Xho*I site adjacent to the ATG start codon and *Sac*I downstream of the TAA stop codon for easy cloning in the forward and reverse primers, respectively (forward, 5′-ctc gag ATG AAG AGA ACA CAT TTG GCA-3′; reverse, 5′-gag ctc TTA GAG GTA GCC TAG TCG AAG-3′). The resulting PCR product was cloned into the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA), and the sequence was verified. The *Xho*I–*Sac*I (in lower-case letters in the primers above) AtZFP11 fragment was excised and cloned into the *Xho*I–*Sac*I site of the pK80-46 35S vector. The entire cassette (−46 35S:AtZFP11:rcbcS T) was taken from the pK80-46 35S vector and cloned into the pCAMBIA 2300 plasmid (CAMBIA, Canberra, Australia) along with the VGCfE$_{VY}$ expression cassette for plant transformation. The resultant binary vector was designated as p2300VGCfE$_{VY}$:AtZFP11 (Fig. 2M). The 35S:AtZFP11:rbcS T cassette taken from the pKYLX80 vector was cloned into pCAMBIA2300 to generate transgenic tobacco and *Arabidopsis* plants that constitutively expressed *AtZFP11* (Fig. 2N). The pCAMBIA2300 binary vector also has the kanamycin resistance gene expression cassette for transgenic plant selection (not shown in Fig. 2).

Transient expression studies

Transient expression studies were carried out by isolating protoplasts from cell suspension cultures of tobacco (*Nicotiana tabacum* cv. Xanthi-Brad). A detailed description of the isolation and electroporation of protoplasts has been given previously [14].

Dose–response study with tobacco protoplasts

The performance of different EcR mutants in inducing luciferase reporter gene activity in the two-hybrid switch format was tested by coelectroporating pK80-46 35S:Luc, pK80VCH9 and pK80GCfE (pK80GCfE$_{Wt}$, pK80GCfE$_{H436E}$, pK80GCfE$_{Q454E}$ or pK80GCfE$_{VY}$) constructs, and the monopartite switch was tested by coelectroporating pK80-46 35S:Luc and pK80VGCfE (pK80VGCfE$_{Wt}$, pK80VGCfE$_{H436E}$, pK80VGCfE$_{Q454E}$ or pK80VGCfE$_{VY}$). Electroporated protoplasts were resuspended in 1 mL of growth medium containing different concentrations of methoxyfenozide, 0 (dimethylsulfoxime control), 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM. Methoxyfenozide stock solutions were made in dimethylsulfoxime and diluted 1000-fold in protoplast growth medium. At 24 h after addition of ligands, the protoplasts were collected by centrifugation and lysed in 100 μL of 1× passive lysis buffer (Promega Corporation). Twenty microliters of protoplast lysate was loaded into each well of a 96-well plate, and luciferase activity was measured in a plate

reader Luminometer (Fluroscan Ascent FL Thermo labsystem, Milford, MA, USA), using a luciferase assay system (Promega Corporation). The protein content in the protoplast extract was measured using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Plant tissue culture

A. thaliana (L.) Heynth. ecotype Columbia ER and *N. tabacum* variety KY160 (University of Kentucky Tobacco Breeding Program) were used for plant transformation experiments. The binary vectors constructed for plant transformation were mobilized into *Ag. tumefaciens*, strain GV3850, by the freeze–thaw method. *Arabidopsis* plants were transformed using the whole plant-dip method [42]. Transgenic *Arabidopsis* plants were selected by germinating the seeds collected from the infiltrated plants on a medium containing 50 $mg \cdot L^{-1}$ kanamycin. Resistant T_1 plants surviving on kanamycin-containing medium were transferred to soil and then moved to a greenhouse for further analysis. Tobacco plants were transformed by employing standard leaf disk transformation protocols and media recipes [43]. The analysis of transgenic plants for luciferase and AtZFP11 induction levels was carried out on T_2 generation lines. The transgenic lines used in all the experiments were screened on kanamycin-containing medium.

Dose–response study with T_2 *Arabidopsis* plants generated for the p2300GCfE$_{VY}$:VCH9:Luc and p2300VGCfE$_{VY}$:Luc constructs

Seeds collected from five T_2 *Arabidopsis* lines were plated on agar medium containing 50 $mg \cdot L^{-1}$ kanamycin and different concentrations of methoxyfenozide (0, 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM). Seeds were allowed to germinate and grow on this medium for 20 days at 25 °C, under 16 h of light and 8 h of dark. Three seedlings from each plate were collected separately and ground in 100 μL of 1× passive lysis buffer (Promega Corporation), and luciferase activity was measured.

Dose–response study with T_2 *Arabidopsis* and tobacco plants generated for the p2300VGCfE$_{VY}$:AtZFP11 construct

Seeds collected from the T_2 *Arabidopsis* and tobacco plants were plated on agar media containing appropriate amounts of kanamycin and different concentrations of methoxyfenozide (0, 0.64, 3.2, 16, 80, 400, 2000 and 10 000 nM). The seeds were allowed to germinate and grow on the induction media for 20 days in the case of *Arabidopsis* and for 4 weeks in the case of tobacco, at 25 °C, under 16 h of light and 8 h of dark.

Microscopy

The transgenic *Arabidopsis* seedlings expressing *AtZFP11* under the VGCfE$_{VY}$ switch were placed on a glass slide and viewed under a Zeiss Stemi SV11 stereo microscope attached to a transilluminating base (Diagnostic Instruments, Sterling Heights, MI, USA). Photographs were taken using an AxioCam MRc 5 camera that was attached to the microscope. Image analysis was carried out with AXIOVISION 4.1 software, and collages were mounted using PHOTOSHOP (Adobe Systems, Inc., San Jose, CA, USA).

qRT-PCR

The expression levels of *AtZFP11* in transgenic tobacco and *Arabidopsis* plants were estimated by qRT-PCR, using SYBR Green I [44]. Total RNA was isolated from 100 mg of tobacco and *Arabidopsis* seedlings using 1 mL of TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). The total RNA isolated using TRIzol reagent was purified by running the samples through Qiagen columns (RNeasy Plant Mini Kit; Qiagen Inc., Valencia, CA, USA) combined with an on-column DNase digestion (RNase-Free DNase set; Qiagen Inc.) to ensure DNA-free RNA preparations. First-strand cDNA was synthesized using the StrataScript First Strand synthesis system (Stratagene, Cedar Creek, TX, USA). DNase-treated RNA samples were tested for genomic DNA contamination by using the minus reverse transcriptase (–RT) controls in parallel with qRT-PCR reactions.

Real-time PCR quantification of the *AtZFP11* transcript was performed by designing specific oligonucleotide primers using PRIMERQUEST software (Integrated DNA Technologies, Coralville, IA, USA) to amplify a 165 bp fragment (forward, 5′-TCC CAT GGC CTC CCA AGA ATT ACA-3′; reverse, 5′-GGT TTG CAA TAG GTG TGT GGT GGT-3′). PCRs were carried out in an iCycler iQ detection system (Bio-Rad Laboratories), using SYBR Green I to monitor dsDNA synthesis. Serial dilutions (10^{-3}–10^{2} $pg \cdot \mu L^{-1}$) of the control plasmid (*AtZFP11* cloned in pGEM-T Easy vector) were used as an external control to generate a standard curve. For negative controls, the cDNA samples of wild-type untransformed tobacco and *Arabidopsis* and DNase-treated – RT controls were used. Real-time PCR amplification was performed in a total volume of 20 μL of reaction mixture containing 1 μL of cDNA or control plasmid, gene-specific primers, SYBR Green I (Molecular Probes, Eugene, OR, USA) and Platinum *Taq* DNA polymerase (Invitrogen, Life technologies). Each sample was loaded in triplicate, and the experiments were repeated twice using the following thermal cycling program conditions: initial denaturation for 2 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C for 35 cycles; and a 5 min extension at 72 °C.

Melt curve analysis [45] was done to characterize the gene-specific dsDNA product by slowly raising the temperature (0.2 °C·10 s^{-1}) from 60 °C to 95 °C, with fluorescence data being collected at 0.2 °C intervals. The starting amount of the *AtZFP11* transcript in each sample was calculated using a standard curve (logarithm of the starting quantity versus threshold cycle) generated for AtZFP11–pGEM-T Easy plasmid dilutions by the iCylcer iQ Optical System Software (Bio-Rad Laboratories).

In order to compare the *AtZFP11* transcript levels from different transgenic plants, the average starting quantity of *AtZFP11* was normalized to the average starting quantity of the α-tubulin gene, which is assumed to be at a constant levels in all the samples. The *Arabidopsis* (forward, 5′-AAG GCT TAC CAC GAG CAG CTA TCA-3′; reverse, 5′-ACA GGC CAT GTA CTT TCC GTG TCT-3′) and tobacco (forward, 5′-ATG AGA GAG TGC ATA TCG AT-3′; reverse, 5′-TTC ACT GAA GGT GTT GAA-3′) α-tubulin-specific primers amplified a 108 bp and a 240 bp fragment, respectively.

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